

## SNAT2 transceptor signalling via mTOR: A role in cell growth and proliferation?

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## 1. ABSTRACT

We have investigated the effect of chronic competitive inhibition of SNAT2 (System A) amino acid (AA) transport, induced by incubation with a saturating dose of a non-metabolisable System A amino acid analogue (Me-AIB), on growth and proliferation of MCF-7 human breast cancer cells in complete culture medium. These cells express Na<sup>+</sup>- and pH-dependent SNAT2 AA transport and a saturating concentration of Me-AIB (10 mM) competitively inhibits (>90%) AA uptake *via* SNAT2. Incubation with Me-AIB for up to 5 days progressively reduced cell proliferation (~2-fold) and depleted intracellular concentrations of not only SNAT2 AA substrates but of essential branched chain AAs (e.g. leucine). Surprisingly, total cellular protein was maintained and cells subjected to chronic Me-AIB incubation exhibited a detectable increase in cell size. Analysis of mTOR signalling revealed that, despite a substantial reduction in size of the intracellular AA pool, Me-AIB elevated mTOR-dependent p70S6K1 phosphorylation. Proteomic analysis of TAP-tag purified SNAT2 fusion proteins identified two novel SNAT2-interacting proteins that may potentially function in conjunction with the SNAT2 transceptor to regulate signalling pathways influencing protein turnover and cell growth.

## 2. INTRODUCTION

Amino acid transporters of the SLC38 family mediate the Na<sup>+</sup>-dependent uptake of small neutral amino acids including key intermediary metabolites (e.g. alanine, glutamine and serine) as well as the indispensable amino acids methionine and threonine. This transporter family (classically known as System A) is comprised of three isoforms, SNAT1, 2 and 4, of which SNAT2 (SLC38A2) is the most widely expressed (1). A characteristic feature of the SLC38 family is their ability to tolerate *N*-methylated amino acid substrates, such as Me-AIB, a property that has proved invaluable for their functional characterisation. The SNATs couple the uphill transfer of amino acids to the inward movement of Na<sup>+</sup> down its electrochemical gradient, hence these transporters develop an outwardly-directed concentration gradient of amino acid substrates which facilitates exchange uptake of a range of indispensable neutral amino acids through transporters (such as System L) arranged in parallel with System A in the plasma membrane (2,3). This type of coupling is critical for both nutrient uptake and nutrient sensing upstream of mTORC1 (the mammalian target of rapamycin complex 1) and it is therefore perhaps unsurprising that SNAT2 is one of the most extensively regulated amino acid transporters known, being responsive to mitogens (including insulin

(4)), amino acid supply, glucocorticoids and cell volume (see reviews (1,5)). In addition, activity of System A is upregulated in contracting skeletal muscle (6), in liver during prolonged fasting to become the rate-determining step in hepatic alanine metabolism (for gluconeogenesis / ureagenesis) (7), and within specific brain regions closely associated with the central response to dietary depletion of single amino acids (8). The available body of evidence thus indicates that System A is a crucial player in the control of diverse amino acid-dependent processes that impact on cell, tissue and whole body function.

Given the functional importance of mTORC1 with respect to regulating cell growth and proliferation, there has been considerable interest over the past decade in defining how supply of nutrients, in particular that of amino acids, promote its activation (9). Very recent work has suggested that amino acids promote localisation of mTORC1 to lysosomal membranes where Rag-GTPases facilitate its interaction with its activator, Rheb (10). Consequently, the activity of membrane amino acid transporters could potentially play a key role in determining the magnitude of mTORC1 activation *via* their ability to regulate both the delivery and size of the free intracellular amino acid pool. However, whilst some transporters may simply function as conduits for delivery of amino acids across the plasma membrane of cells, there is a growing body of evidence that suggests that others may function as “transceptors” capable of integrating amino acid sensing/signalling functions with their role as transporters (reviewed in (3)). Amino acid uptake *via* a number of these transceptors is rheogenic and thus the ionic current generated as part of the transport cycle may, in some cases, be of sufficient magnitude to elicit activation of voltage-sensitive signalling mechanisms (11,12) that could stimulate mTORC1 (13). Equally, impaired transporter expression/function may have implications for regulation of mTORC1. There is in fact evidence that a reduction in SNAT2 activity in L6 myotubes is associated with impaired mTORC1 activation (14), and more recent work, involving RNA knockdown of proton-assisted amino acid transporters (PATs), has identified PAT1 and PAT4 as being a requirement for supporting the activation of mTORC1 in human MCF-7 breast cancer cells (15). The precise mechanism(s) by which changes in transporter expression/function impact on mTORC1 activation remains poorly understood, but it is plausible that substrate binding to amino acid transceptors, such as SNAT2, may either induce and/or affect the activity of associated proteins capable of intracellular signalling, as has been shown for a number of transceptors (e.g. Ssy1 and Gap1) in lower eukaryotes (16,17). In this study we have assessed (i) the effects of chronic cell exposure to saturating doses of Me-AIB (as a means of inhibiting uptake of natural System A substrates) upon growth and proliferation of human MCF-7 breast cancer cells, (ii) investigated SNAT2 associations with intracellular proteins and (iii) determined whether mTOR signalling is affected by SNAT2 substrate supply.

### 3. MATERIALS AND METHODS

#### 3.1. Materials

Culture media (Dulbecco's modified Eagle's medium, DMEM (Catalog number 12800-017);  $\alpha$ -minimal

essential media,  $\alpha$ -MEM, Catalog number 11900-016), foetal calf serum, and antimycotic/antibiotic solution were obtained from Invitrogen, Life Technologies (Paisley, Renfrewshire, Scotland). Precise media formulations can be accessed using the catalog numbers from the Invitrogen website (<http://www.invitrogen.com>). Insulin, rapamycin and  $\alpha$ -methylaminoisobutyric acid were purchased from Sigma-Aldrich Co. Ltd. (Poole, England). [ $^{14}$ C]-Me-AIB ( $\alpha$ -methylaminoisobutyrate) was from PerkinElmer Life Sciences. Antibodies to phospho p70S6K and S6 protein were obtained from New England Biolabs (Hertfordshire, UK). Horseradish peroxidase conjugated anti-rabbit IgG was obtained from Scottish Antibody Production Unit (Carlisle, Lanarkshire, Scotland). Hybond nitrocellulose membrane was obtained from Amersham Life Sciences, (Amersham Pharmacia Biotech, UK) and reagents for ECL were purchased from Pierce & Warriner (Chester, UK). TAP tag vectors (C-terminal and N-terminal) were purchased from Promega

#### 3.2. Cell Culture

MCF-7 breast adenocarcinoma cells and human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 10% fetal calf serum and glutamine. L6 muscle cells were cultured to the stage of myotubes as described previously (18,19) in  $\alpha$ -MEM containing 2 % (v/v) foetal calf serum and antimycotic/antibiotic solution (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 250 ng/ml amphotericin B). All cell lines were maintained normoxically in 5% CO<sub>2</sub> at 37°C and 95% humidity and treated as indicated in the figure legends.

#### 3.3. Analysis of cell number, size and intracellular amino acids

MCF-7 cells were plated at a density of  $1 \times 10^5$ /well in 6-well plates in triplicate and maintained in growth media containing foetal calf serum in the absence or presence of Me-AIB for periods indicated in the figure legends. At the end the appropriate period of incubation, cells were washed in HEPES-buffered saline and exposed to 0.4% (w/v) trypan blue dye solution; the number of viable cells, based on trypan blue exclusion, were counted. For analysis of cell size we used flow cytometry:- cells were trypsinised, stained with propidium iodide and washed before analysis on a FACSCalibur (Becton Dickinson, San Jose, CA). Cells were gated according to their forward light scatter (FSC) and side light scatter (SSC) profiles. For analysis of intracellular amino acids, MCF-7 cells were grown on 3.5 cm plates and cultured in DMEM media supplemented with either 10 mM Me-AIB or 10 mM sucrose (control) for 5 days. Following this treatment period, MCF-7 cells were washed twice with ice cold PBS and scraped into 300 mM perchloric acid. The suspension was centrifuged (3,000 g, 10 min) and the supernatant was neutralised with KOH and centrifuged (13,000 g, 10 min). The amino acid containing supernatant was dried in a vacuum concentrator, analysed using the PICO-TAG high performance liquid chromatography system (Waters Corporation, Milford, MA, (20)) and standardised to protein recovery.

### 3.4. SDS-PAGE and immunoblotting

Cell lysates (50 µg protein) were subjected to SDS-PAGE and immunoblotting as described previously (18). Separated proteins were transferred onto nitrocellulose membranes and blocked with Tris-buffered saline (pH 7.0) containing 5% BSA and 0.05% (v/v) Tween-20. Membranes were probed with anti-phospho p70S6K and anti-S6 (both used at a final dilution of 1:1000) antibodies. Following primary antibody incubation, membranes were washed then incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:1000). Immunoreactive protein bands were visualised by enhanced chemiluminescence on Konica Medical film (Hohenbrunn, Germany).

### 3.5. Measurement of System A amino acid transport

System A activity was assayed by measuring the uptake of methyl-aminoisobutyrate (Me-AIB) as described previously (19). Briefly, MCF-7 cells were incubated in Hepes/NaCl (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub> and 5 mM glucose, pH 7.5) containing 10 µM [<sup>14</sup>C]-Me-AIB (1 µCi/ml) for 20 min. Non-specific tracer binding was assessed by determining cell associated radioactivity in the presence of a saturating dose of unlabelled Me-AIB (19). Me-AIB transport kinetics were determined by assaying [<sup>14</sup>C]-Me-AIB uptake over a range of substrate concentrations (between 10 µM and 10 mM). V<sub>max</sub> and K<sub>m</sub> were determined by the Lineweaver-Burk method with regression analysis performed using GraphPad Prism software. pH and ion dependence were assayed by altering pH of the uptake assay solution or replacing NaCl with an equivalent concentration of tetramethylammonium chloride. Uptake of Me-AIB was determined by aspirating the radioactive medium, followed by three successive washes in ice cold isotonic saline solution (0.9% NaCl, w/v). Cells were lysed in 0.05 M NaOH and the associated radioactivity determined by liquid scintillation counting. Total cell protein was determined by the method of Bradford (21).

### 3.6. Proteomic analysis of TAP-tagged SNAT2 complexes

For analysis of SNAT2 interacting proteins we adopted the tandem affinity purification (TAP) tag strategy that allows rapid purification of protein complexes (22) and which, when combined with mass spectrometry, allows identification of interacting proteins. We generated a TAP-tagged SNAT2 gene construct by cloning of rat SNAT2 (23) with a modified TAP tag (pEGFP-2C-TAP (24)) adjacent to the 5' end of the gene. This construct (pEGFP-2C-TAP-SNAT2) adds green fluorescent protein (GFP) and a Protein A binding-motif upstream of a calmodulin (CAM) binding-motif flanked by TEV and precision protease cleavage sites, all 5' to SNAT2. The pEGFP-2C-TAP-SNAT2 construct was transfected into HEK293 cells and clonal lines produced by G418 selection. The level of stably-expressed SNAT2 protein produced by this procedure was assessed by immunoblot using an anti-SNAT2 antibody (25). Clones showing relatively low levels of expression were selected for further study, as recommended for the TAP-tag system (highly over-

expressing clones were avoided to minimise the likelihood of masking endogenous interactions). TAP-tagged SNAT2 in cell lysates from these clones was purified by two different rounds of affinity chromatography enabled by the TAP strategy:- (i) IgG-agarose with TEV protease elution (cleaving the EGFP peptide label and Protein A motif), followed by (ii) calmodulin-sepharose with EGTA elution. Two separate full-scale purifications from cell lysates pooled from 50 x 15cm dishes of clonal (pEGFP-2C-TAP-SNAT2)-HEK293 cells were completed and the protein composition of the final eluate was established by polyacrylamide gel electrophoresis with colloidal Coomassie Blue staining. Identification of TAP-purified peptides was undertaken following tryptic digestion and mass-spectrometric fingerprint analysis (24) using the Mascot search algorithm [http://www.matrixscience.com (26)].

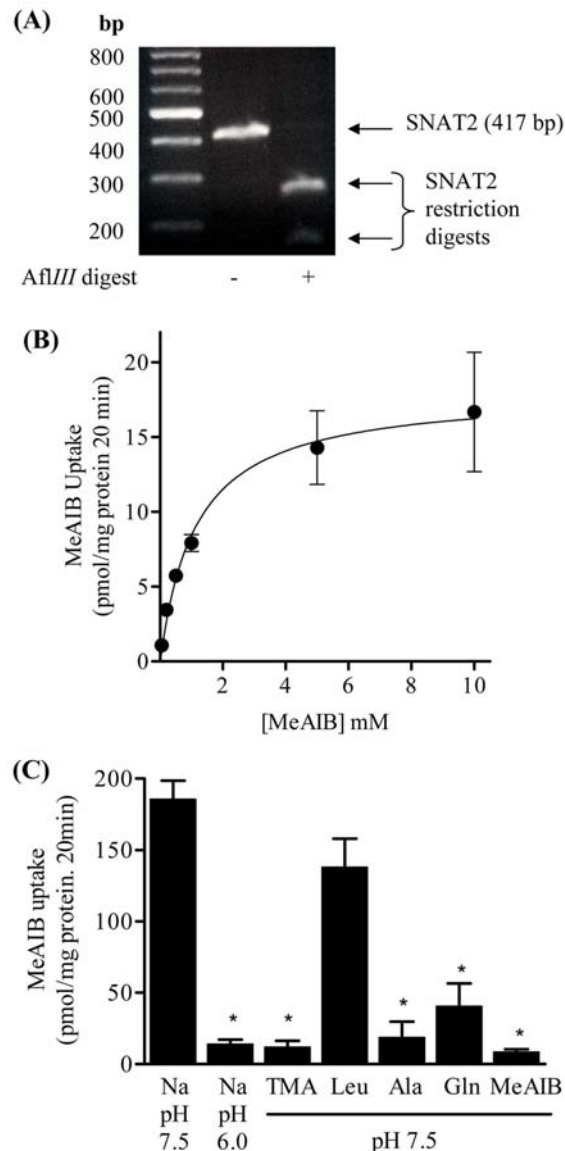
### 3.7. Statistical analysis

For multiple comparisons, statistical analysis was performed using one-way analysis of variance (ANOVA). For individual comparisons, statistical analysis was performed using Student's t-test. Data analysis was performed using GraphPad Prism software and considered statistically significant at P values < 0.05.

## 4. RESULTS

### 4.1. MCF-7 cells express SNAT2 and exhibit functional System A transport

MCF-7 cells are rapidly proliferating breast cancer cells and consequently provide a useful model for assessing whether modulation of System A activity may be an important determinant for growth and proliferation of such cells. However, it was initially important to establish that these cells express SNAT2 and exhibit functional System A transport activity. RT-PCR analysis confirmed that MCF-7 cells express mRNA for SNAT2 (Figure 1A). We subsequently assayed System A transport in MCF-7 cells using labelled Me-AIB; a paradigm substrate for this amino acid transporter. Figure 1B shows that MCF-7 cells exhibit a saturable Michaelis-Menten Me-AIB uptake profile. Regression analysis of the uptake data revealed that the transport maximum (V<sub>max</sub>) was 18.08 ± 0.68 pmol/mg protein.20min and that the Me-AIB concentration at which this was half-maximal (i.e. K<sub>m</sub>) was 1.14 ± 0.15 mM. Consistent with the well established ion and pH dependence of System A, Figure 1C shows that uptake of 10 µM Me-AIB was depressed markedly when transport buffer was either acidified (pH 6) or in which sodium chloride was replaced with tetramethylammonium (TMA) chloride. Uptake of 10 µM [<sup>14</sup>C]-Me-AIB was also reduced significantly when the transport assay buffer was supplemented with a competing dose (10 mM) of unlabelled alanine, glutamine and Me-AIB; a finding in line with the idea that these amino acids are taken up into MCF-7 cells *via* a common transporter (i.e. SNAT2). In contrast, addition of 10 mM leucine (a System L substrate) to the uptake buffer did not induce a significant inhibition in [<sup>14</sup>C]-Me-AIB uptake.



**Figure 1.** SNAT2 expression and functional System A transport in MCF-7 cells. (A) RNA was prepared from confluent MCF-7 cells, cDNA synthesised and PCR carried out using primers specific to SNAT2. AFLIII digest of the 417bp PCR product generated two smaller fragments of the expected size, confirming identity of the amplicon as the appropriate SNAT2 fragment. (B) Analysis of Me-AIB transport was performed by assaying [ $^{14}\text{C}$ ]-Me-AIB uptake over a range of concentrations as shown. (C) pH and Na dependence of System A transport in MCF-7 cells was assayed as described in methods, the competitive effect of leucine (Leu), alanine (Ala) and glutamine (Gln) on Me-AIB uptake was assessed by having these amino acids present in the uptake solution at a concentration of 10 mM. Values in B and C are mean + SEM for up to 4 separate experiments. Asterisks signify a significant change ( $P < 0.05$ ) from the control uptake bar (Na, pH 7.5).

#### 4.2. Effects of sustained competitive-inhibition of System A on cell growth and proliferation

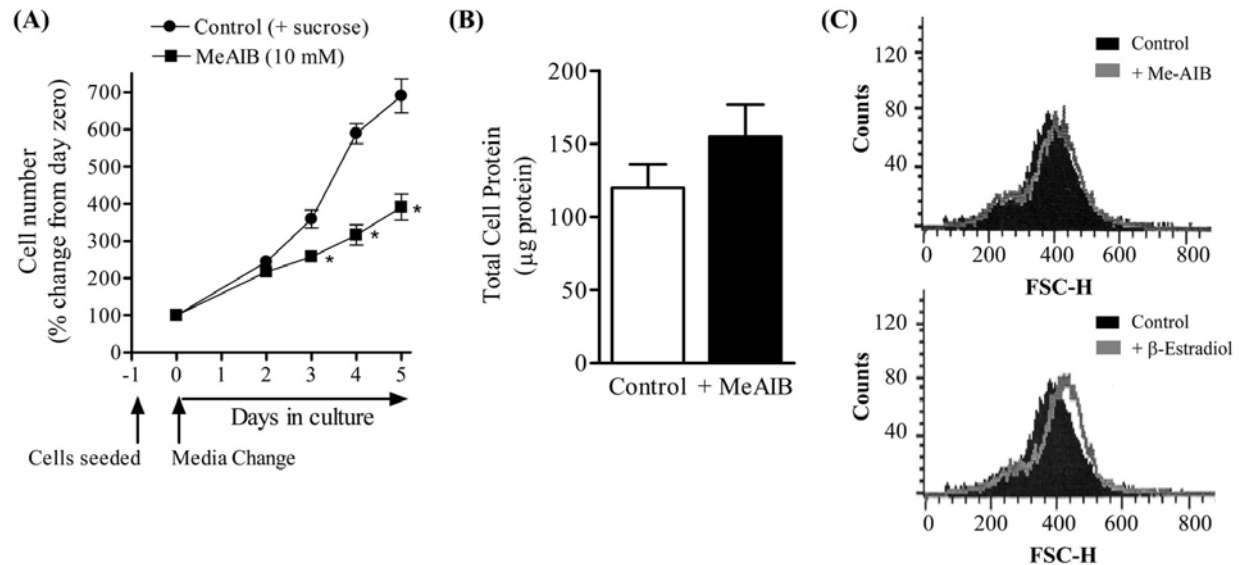
Since Me-AIB is not metabolised, but is capable of inducing a substantial reduction in the uptake of native System A substrates, we assessed the effect of chronic incubation of MCF-7 cells with Me-AIB at concentrations that would saturate SNAT-mediated transport. MCF-7 cells were seeded ( $10^5$  cells) into culture wells and maintained in standard DMEM for an initial 24h. At the end of this period, media was refreshed and supplemented with either 10 mM sucrose (control) or 10 mM Me-AIB and maintained in culture for up to 5 days. Cells were harvested at regular periods during this time and cell number and total protein assessed. Figure 2A shows that, within 3 days of adding Me-AIB to the culture medium, proliferation of MCF-7 cells was noticeably reduced such that by day 5 there was nearly a 2-fold difference in cell number between the control and Me-AIB treatments. Surprisingly, however, despite the significant reduction in cell number, analysis of total cell protein revealed a marginal increase in cells treated with Me-AIB compared with control cells, although this was not statistically significant (Figure 2B). To test whether differences in cell size could account for lack of difference in total cell protein, we performed flow cytometry on control and Me-AIB treated cell populations. This analysis indicated that, whilst Me-AIB reduced proliferation of MCF-7 cells, treatment with this amino acid analogue had induced an increase in cell size by ~8% compared to the untreated control cell population (Figure 2C). Treatment of MCF-7 cells with 10 nM  $\beta$ -estradiol, a hormone stimulus known to positively enhance growth of MCF-7 (27) induced a marginally higher, but comparable increase in cell size (~10%) based on FACS forward-scatter analysis.

#### 4.3. Chronic cell incubation with Me-AIB and intracellular amino acid levels

To investigate the impact of sustained System A inhibition in MCF-7 cells on the free intracellular concentration of SNAT2 substrate amino acids, we performed HPLC analysis of cell lysates. Figure 3 shows that, with the exception of glycine, a 5 day period of cell incubation with Me-AIB induced a significant decline in the free intracellular concentration of key SNAT2 substrates (i.e. serine, glutamine, alanine, threonine), but also that of amino acids that serve as substrates for System L (leucine, isoleucine, valine and phenylalanine).

#### 4.4. System A-induced modulation of mTOR signalling

Since maintenance of cellular protein mass in the face of both reduced amino acid delivery and a diminishing free intracellular amino acid pool implied adaptive changes in protein turnover (i.e. synthesis and breakdown of protein), we assessed the activation status of p70S6K, a key signalling intermediate downstream of the mTORC1 complex involved in regulating components of the mRNA translation machinery and also autophagy (9). Immunoblotting MCF-7 cell lysates following sustained incubation with 10 mM Me-AIB revealed that these cells exhibit elevated p70S6K1 phosphorylation relative to that observed in control cells. Cell treatment with rapamycin 30 min prior to lysis led to a complete loss in basal and Me-



**Figure 2.** Effects of sustained MCF-7 cell incubation with Me-AIB upon cell proliferation, total cell protein and cell size. (A) MCF-7 cells were incubated with 10 mM Me-AIB in complete medium for periods indicated and adherent cell number quantified by analysis of trypan blue exclusion. (B) Cells incubated in culture  $\pm$  Me-AIB for 5 days were harvested and total protein assessed using the Bradford method. (C) Cells cultured in the absence or presence of 10 mM Me-AIB or 10 nM  $\beta$ -estradiol for 5 days were harvested for flow cytometry analysis to gauge effect of treatments on cell size. FSC-H; Forward scatter-height. Values in A and B are mean  $\pm$  SEM for up to 4 separate experiments. Asterisks signify a significant change ( $P < 0.05$ ) from the corresponding cell count obtained for cells not incubated with Me-AIB.

AIB-induced phosphorylation, consistent with the idea that heightened p70S6K1 phosphorylation was attributable to elevated mTOR activity (Figure 4A).

To test whether the Me-AIB dependent activation of mTOR/p70S6K1 signalling observed in MCF-7 cells may have reflected a cell-specific effect, we subsequently assessed whether acute provision of Me-AIB to differentiated L6 rat skeletal muscle myotubes would stimulate p70S6K1 phosphorylation. As positive controls, the effect of insulin and leucine (a branched chain amino acid (BCAA) widely regarded as exerting a potent stimulatory effect on the mTOR pathway (9)) were also tested. Figure 4B shows that L6 myotubes maintained in  $\alpha$ -MEM containing amino acids exhibit high basal p70S6K1 phosphorylation. In order to enhance the sensitivity of detection and to establish whether Me-AIB, a SNAT2 substrate, could acutely stimulate p70S6K1, muscle cells were amino acid deprived for 4h by incubation in HEPES-buffered saline containing 5 mM glucose. This intervention led to a significant lowering in basal p70S6K1 phosphorylation (lane 2). Subsequent incubation of cells held in amino acid-free buffer with just 2 mM leucine (a concentration that closely equates to the total BCAA concentration present in DMEM/ $\alpha$ -MEM culture media) for 30 min induced a significant increase in p70S6K1 (lane 3), which was further enhanced when muscle cells were co-treated with insulin (lane 4). Strikingly, incubation of amino acid-deprived muscle cells with 2 mM Me-AIB alone also induced p70S6K1 phosphorylation (lane 6), an effect which was also additive to the insulin response (lane 7). Activation of p70S6K1 in response to both leucine and

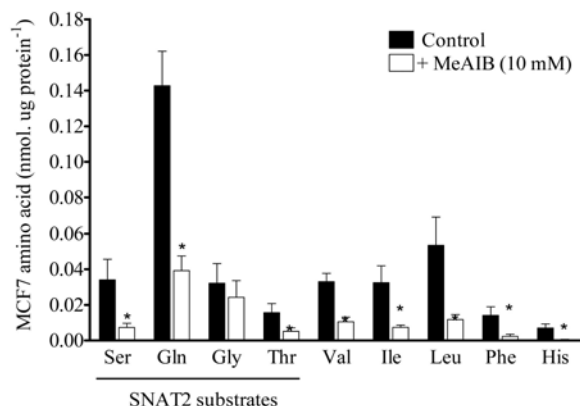
Me-AIB was mTOR-mediated, based on their respective sensitivity to rapamycin (lanes 5 and 8).

#### 4.5. Analysis of TAP-tagged SNAT2 protein complexes

To assess whether we could identify novel SNAT2 interacting proteins that may potentially participate in SNAT2 transceptor-associated signalling, we analysed SNAT2 protein complexes isolated by the TAP-tag strategy. Figure 5 shows an immunoblot confirming expression of the pEGFP-2C-TAP-SNAT2 construct in HEK293 cells using an antibody against a N-terminal SNAT2 epitope (25). It should be noted that the GFP-TAP peptide tag adds considerably to the molecular size of the SNAT2-fusion protein and consequently it migrates at  $\sim$ 150 kDa on SDS-PAGE gels. Intriguingly, in both the initial HEK293 parent line that we transfected and in two separate cell clones that we subsequently propagated, we also observed a faster migrating TAP-SNAT2 immunoreactive band of  $\sim$ 120 kDa. Purification of SNAT2 fusion protein from HEK293 cell lysate using the TAP approach yielded four proteins identifiable by nLCMS analysis of tryptic fragments from polypeptide bands separated by SDS PAGE. These proteins were SNAT2 itself (identified from 2 N-terminal tryptic peptides), myosin light-chain kinase II (MLCK II), ribosomal protein s25 and tubulin (Table 1). Of these, tubulin co-purifies with other TAP-tagged fusion proteins expressed in HEK293 cells and may be a non-specific contaminant (24).

## 5. DISCUSSION

Growth and proliferation of cells is dependent upon an adequate supply of nutrients (glucose, amino acids,



**Figure 3.** Effects of sustained MCF-7 cells incubation with 10 mM Me-AIB in complete medium on intracellular concentration of System A and L amino acid substrates. Amino acid values are mean  $\pm$  SEM from 3 separate experiments. Asterisks signify a significant change ( $P < 0.05$ ) from the untreated control.

fatty acids etc) to support both the biosynthesis of a diverse set of macromolecules (including proteins, nucleic acids and complex lipids) and the energy demands associated with the processes of growth and renewal. Amino acids are of particular interest, because they not only serve as precursors for protein synthesis but are known to exert powerful regulatory effects on cellular function (28). For mammalian cells there is considerable evidence that altered amino acid supply directly influences signal transduction and gene expression events, as well as altering the cellular responses to hormonal stimuli. Several reviews (9,28-30) have summarised current knowledge of the signalling pathways and transcriptional-translational regulators responsive to nutrients, but the relative importance of specific amino acid transporters and of extracellular versus intracellular amino acid provision in the control of growth and proliferation still remain poorly defined. The objective of the present study was to establish the importance of amino acid delivery *via* the System A transporter for growth and proliferation of MCF-7 cells; a well established adenocarcinoma cell line that exhibits strong potential for growth and proliferation in culture and one that has been used extensively for *in vitro* breast cancer studies (31). Our findings indicate that MCF-7 cells express the SNAT2 System A transporter and that sustained inhibition of natural amino acid uptake from complete culture medium *via* this transporter, using a competing saturating dose of Me-AIB, exerts a strong repressive effect on the proliferative capacity of MCF-7 cells. Very surprisingly, despite the reduced cell count associated with long-term inhibition of System A, we did not observe any reduction in total protein in cells chronically incubated with Me-AIB compared with the proliferating control cell population. This discrepancy appears to be explained, at least in part, by the finding that average size of cells treated with Me-AIB is larger than that of those not exposed to Me-AIB treatment, perhaps implying that cell growth is less restrained than cell proliferation.

Competitive inhibition of System A transport by MeAIB treatment is associated with a substantial reduction in the intracellular concentrations of SNAT2 amino acid substrates but also, significantly, of essential amino acids such as the branched chain amino acids (BCAA; leucine, isoleucine, valine), which serve as substrates for System L (but not System A). Such reductions are likely to be attributable to two factors:-

**Firstly;** inhibition of SNAT2/System A activity will reduce the contribution made by its substrates to the “free” intracellular amino acid pool. Indeed, previous work has shown that silencing SNAT2 expression in human fibroblasts similarly induces a significant lowering of intracellular amino acids (32). In consequence, given that several SNAT2 substrates are involved in tertiary heteroexchange *via* System L, reduced intracellular accumulation of SNAT2 substrates will also suppress the counter-drive for take up of System L amino acids (*e.g.* the BCAA) (2,3).

**Secondly;** the “free” intracellular amino acid pool will also be subject to “drainage” as it continues to provide precursors to help maintain protein synthesis in support of cell growth. However, our findings indicate that a contracting intracellular amino acid pool is unable to sustain cell proliferation; a process which, we therefore suggest, is more dependent on the efficient supply of extracellular amino acids. This supposition is consistent with very recent work showing that supply of extracellular glutamine (a SNAT2 substrate) is pivotal for inducing activation and proliferation of T-lymphocytes (33).

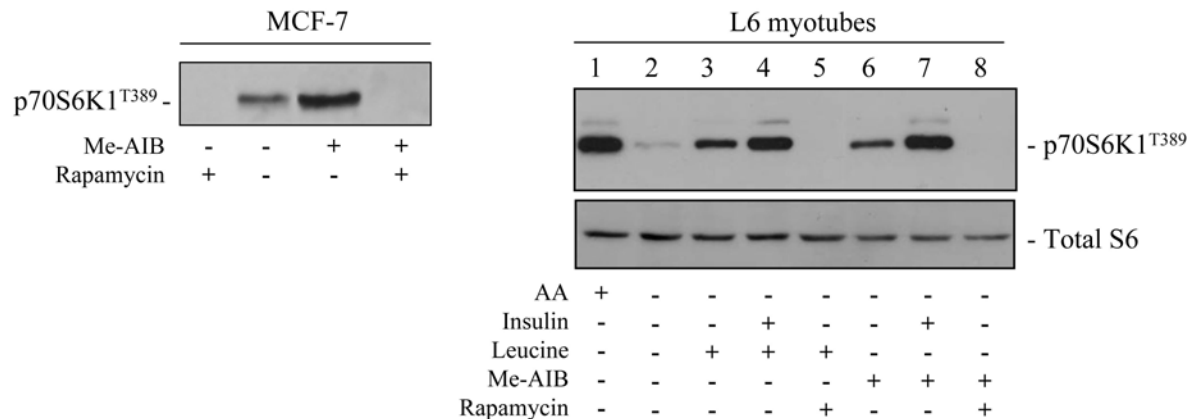
Tissue protein synthesis is known to be modulated in response to changes in nutritional status. For example, overnight fasting of experimental animals is known to reduce blood amino acid concentrations by ~50% relative to the fed state (from 6.1 to 3.0 mM) (34) and is also associated with a significant repression in tissue p70S6K1 phosphorylation and protein synthesis, both of which can be rapidly reversed upon refeeding (35). Likewise, we show here that cells maintained in culture media containing amino acids, which provides some semblance of the *in vivo* “fed” state, exhibit elevated p70S6K phosphorylation. This activation of p70S6K may be reversed by subjecting cells to a short period of amino acid withdrawal to mimic the “starved” or “fasted” state, a manoeuvre which has helped us to unmask the stimulatory potential that Me-AIB possesses for promoting p70S6K activation. Collectively, the *in vivo* and *in vitro* observations help underscore the important role that extracellular amino acids play with respect to tonic stimulation of the mTOR/S6K1 pathway.

How then might cellular protein mass be maintained in the face of reduced amino acid delivery (*via* System A and transporters mechanistically linked to its activity) and a diminishing intracellular amino acid pool? Given that amino acids play a crucial role in the activation of the mTOR/p70S6K signalling axis and also that this pathway regulates key components of the cellular machinery effecting protein turnover, we were keen to

**Table 1.** Results of MASCOT MS/MS ion search on tryptic digest of peptides recovered form TAP-tag purification.

Peptide	Gene	Comments
K.SHYVDVDPENQNFLLESNLG.K	SNAT2 (SLC38A2)	Residues 39-60 P<0.001
K.TANEGGSLLYEQLGH.K	SNAT2 (SLC38A2)	Residues 124-141 P<0.1
K.KNFIASVSAANR.F	MLCK2	P<0.001
K.LITPAVVSERL	Ribosomal protein s25	P<0.05
R.ISEQFTAMFR.R	β tubulin	P<0.05

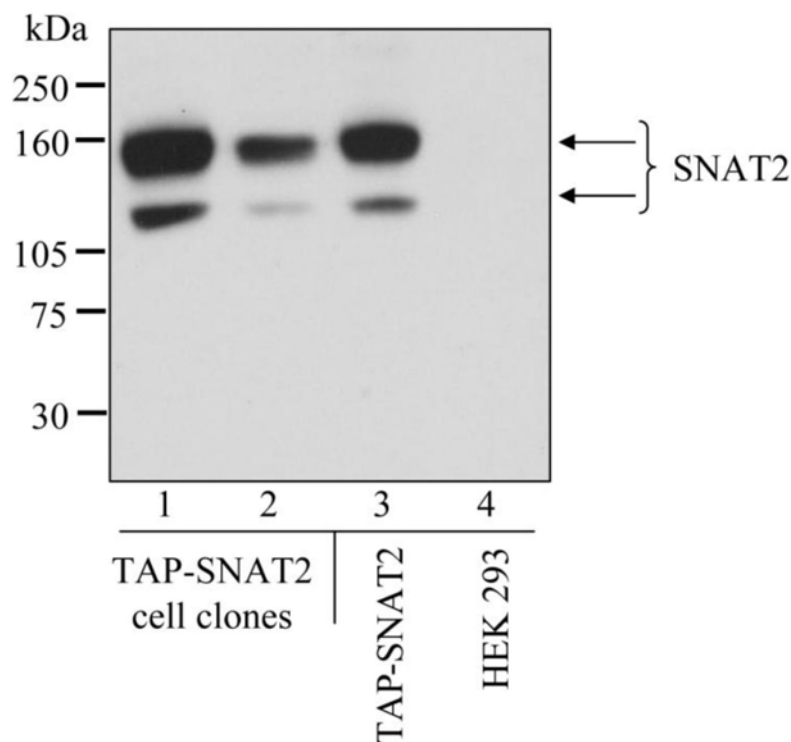
Samples were in-gel reductively alkylated after colloidal coomassie staining, digested with 12.5 µg/ml trypsin and spotted 5.0 µl from 100 µl of extract after an equal addition of ACN for mass fingerprint analysis. Subsequently extracted and analysed by nLCMS (ESI-TRAP)



**Figure 4.** Effects of Me-AIB on p70S6K1 phosphorylation in MCF-7 cells and L6 myotubes. (A) MCF-7 cells were incubated in the absence and presence of 10 mM Me-AIB in complete medium for 5 days after which time cells were lysed and immunoblotted using a phospho-p70S6K1 antibody. L6 myotubes were maintained in complete media containing a physiological amino acid mix or maintained in amino acid free Hepes-buffered saline (HBS) for 4h prior to supplementation with 2 mM leucine or 2 mM Me-AIB for 30 min prior to cell lysis. In some experiments (where indicated), rapamycin (100 nM) was added 30 min prior to addition of either leucine or Me-AIB. Cell lysates were immunoblotted using antibodies to S6 (used as a gel loading control) and phospho-p70S6K1. The blots shown are representative of three separate experiments.

assess what impact Me-AIB would have on mTOR signalling. Our findings indicate that both acute and sustained incubation of cells with Me-AIB results in enhanced activation of mTOR based on observations of a rapamycin-sensitive increase in p70S6K1 phosphorylation. This observation is particularly novel given that Me-AIB is not incorporated into protein or otherwise metabolized, raising the possibility that amino acid substrate-binding to SNAT2 and/or transport across the membrane may lead to initiation of a signalling pathway that culminates in mTOR activation. Although we cannot exclude the possibility that Me-AIB may interact with an intracellular sensor/signalling protein in mammalian cells, previous work from our group has shown that, unlike leucine, microinjection of Me-AIB into the intracellular compartment of amphibian oocytes does not promote mTOR/p70S6K1 activation (36). Assuming this also holds for mammalian cells, then our findings appear to implicate SNAT2 itself in the signal initiation event. The notion that SNAT2 may function as a transceptor is already supported by studies showing that incubation of muscle cells with a saturating dose of Me-AIB may act to synergize the effect of insulin on the mTOR pathway by enhancing PI3-kinase activity and PKB/Akt phosphorylation (37). This synergy is lost upon silencing of SNAT2 gene expression (using RNA interference), which results in a suppressive effect on PI3-kinase (37). Although it remains unclear precisely how SNAT2 may modulate PI3-kinase/PKB signalling, it is

conceivable that the positive effects of Me-AIB upon mTOR signaling may involve interaction and activation of amino acid-sensitive kinases akin to either *Ste20* (38) or *hVps34* (13) with SNAT2. Whilst there is no evidence in the literature to suggest that these particular kinases associate with SNAT2, our TAP-tag purification strategy identified MLCK II as one of three novel SNAT2 interacting proteins. At this stage we cannot entirely exclude the possibility that MLCKII binds through the CAM motif of the TAP-tag, but this seems extremely unlikely on the basis that MLCKII has not been detected in other HEK293-based TAP-tag protein purification protocols. The identification of MLCKII as a putative binding partner for SNAT2 raises the intriguing possibility that it may link transporter-occupancy / transport-flux to signalling pathways regulating cell growth and proliferation. Indeed, inhibition of MLCK (using the inhibitor ML7) completely attenuates the adaptive upregulation of System A in amino acid-deprived rat skeletal muscle cells (Cwiklinski, Taylor & Hundal, unpublished data). Although no evidence currently exists to support a direct link between MLCKII and mTOR signalling, the Ca<sup>2+</sup>-calmodulin dependence of MLCKII fits well with the observation that mTOR activation may be linked to rises in intracellular free Ca<sup>2+</sup> (13). MLCK activity is actually required for plasma-membrane localisation of Ca<sup>2+</sup>-permeable TRPC5 channels (39), as well as for proliferation of coronary artery smooth muscle



**Figure 5.** N-terminal TAP-tag SNAT2 fusion proteins expressed and purified from HEK293 cells were subjected to SDS-PAGE and immunoblotting with SNAT2 antibodies. Analysis was performed in two separate TAP-tag SNAT2 expressing clones of HEK293 cells (lanes 1 and 2) that had been propagated from the original transfected line (lane 3). Untransfected HEK293 cells processed in the same manner as those expressing the TAP-tag SNAT2 were run alongside as a negative control (lane 4).

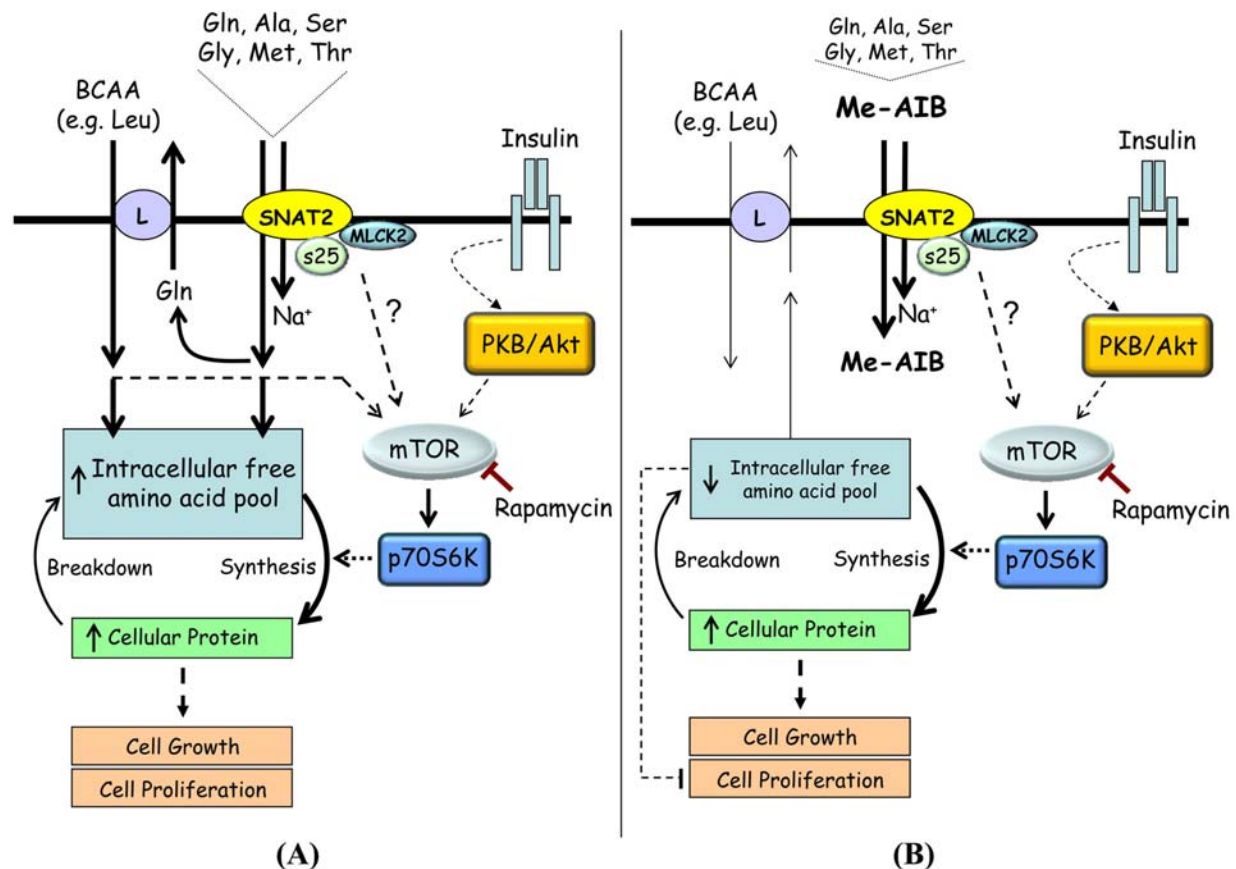
cells (40). The finding that ribosomal protein s25 is associated with SNAT2 is of interest given that, like SNAT2, s25 is one of few genes to be induced in response to cellular amino acid deprivation (25,41). Although the significance of a physical interaction between SNAT2 and s25 is unknown at present, the latter has been implicated in regulating the translation initiation step (42) and may be important for selective upregulation of the synthetic rate of proteins that are needed upon cellular amino acid replenishment (41). The specificity of the putative interaction between SNAT2 and tubulin remains to be confirmed, although it is not inconsistent with previous studies in which a transport activity resembling System A was shown to co-purify with other cytoskeletal proteins (43), as well as with integrin signalling complexes (44).

The finding that TAP-tagged SNAT2 was recovered as two distinct polypeptide fragments is unlikely to be an artefact associated with the TAP purification procedure, based on our previous observation that immunoblotting lysates prepared from muscle cells identifies two protein bands of distinct molecular size (~60 kDa and 35 kDa) that react against antibodies to the N-terminal region of SNAT2 (25). The difference in molecular size of the two native proteins that we detect in whole cell lysates is ~25-30 kDa (25), which is similar to the difference between the two SNAT2 fragments purified using our N-terminal TAP-tag approach (Figure 5);

similarly, SNAT2 labelled with a C-terminal V5 tag is expressed as two peptides of ~60 kDa and 23 kDa (unpublished observations). The abundance of both polypeptides recognised by the N-terminal SNAT2 antibody is enhanced significantly in response to amino acid withdrawal (the larger protein more so than the smaller) suggesting that they are either upregulated independently of each other in response to amino acid limitation or, alternatively, the smaller protein may be generated by proteolytic cleavage of the larger SNAT2 fragment (25). Post-translational proteolytic cleavage is a recognised feature of several membrane-spanning proteins including the  $H^+$ -gated acid-sensing ion channel (ASIC1) (45) and the  $Na^+$  channel, ENaC (46). ENaC proteolysis appears to be an important means of channel activation (46), raising the fascinating possibility that a selective proteolytic processing event may also be involved in rendering the SNAT2 transceptor functionally competent; testing this possibility represents an important investigative goal for future work.

In summary (Figure 6), our ongoing work indicates that maintaining functional System A transport across the plasma membrane is required to support the efficient proliferation of cells (at least MCF7 cells) in culture. Sustained inhibition of System A induces a significant depletion of the intracellular amino acid pool which, despite not being replenished by supply of





**Figure 6.** Schematic illustrating (A) the potential roles of SNAT2 in regulation of System L transport *via* heteroexchange, contribution of substrates to the intracellular amino acid pool and regulation of protein synthesis / cellular proliferation *via* mTOR and p70S6K. (B) A saturating dose of Me-AIB suppresses uptake of native SNAT2 substrates, which impacts on uptake of System L amino acids resulting in a reduced intracellular amino acid pool. Binding of Me-AIB to SNAT2 (or to an unknown intracellular sensor) may modulate activity of SNAT2-associated proteins (*e.g.* MLCKII) to upregulate mTOR signalling and stimulate net protein synthesis sufficiently to support cell growth. The reduction in amino acid delivery to the cytosol (and consequent decrease in the intracellular amino acid pool) is unable to sustain normal rates of cell proliferation.

extracellular amino acids, appears sufficient to sustain continued cell growth but insufficient to maintain normal proliferative rate. Our data also indicate that, whilst chronic inhibition of System A with Me-AIB induces significant disturbances in cellular amino acid nutrition, the drive for maintaining protein synthetic capacity for cell growth (and a concomitant reduction in autophagy) may, in part, be attributed to enhanced activation of the mTOR/p70S6K signalling axis initiated by Me-AIB binding to the SNAT2 transceptor. This scenario differs from typical amino acid starvation by the fact that it is achieved by providing a saturating concentration of a non-metabolisable competitive transport-inhibitor (rather than by direct depletion of extracellular amino acids), which appears to at least partly maintain the mTOR signal for cell growth. The results also provide an interesting addition to the body of data relating mammalian cell size to cell proliferation rate (see (47) for recent perspective), by showing that mammalian cells may increase in size even under circumstances when proliferation appears to be suppressed.

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**Abbreviations:** Methyl aminoisobutyrate, Me-AIB; mTORC, mammalian target of rapamycin complex; TAP, tandem affinity purification; MLCK, myosin light chain kinase; BCAA, branched chain amino acids.

**Key Words:** p70S6K1, System A, Me-AIB, Leucine, BCAA, Protein Synthesis, MCF-7 cells, L6 Myotubes, Muscle

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